Identification of pyroglutamyl peptides with anti-colitic activity in Japanese rice wine, sake, by oral administration in a mouse model

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Title: Identification of pyroglutamyl peptides with anti-colitic activity in Japanese rice wine, sake, by oral administration in a mouse model.

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Abstract: Two pyroglutamyl peptides with anti-colitic activity were identified in Japanese rice wine, sake, by oral administration of a small dose (0.1-1.0 mg/kg body weight) and in vivo activity-guided fractionation. Compounds in sake were fractionated by preparative isoelectric focusing followed by preparative reversed phase-liquid chromatography. Anti-colitic activity was evaluated using a dextran sulfate sodium (DSS)-induced colitis mouse model. The final active fraction contained three pyroglutamyl peptides; pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile) and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-proline (pyroGlu-Asn-Ile-Asp-Asn-Pro). Orally administrating artificially synthesized pyroGlu-Tyr (1.0 mg/kg body weight) and pyroGlu-Asn-Ile (0.1 and 1.0 mg/kg body weight) exhibited a significant protective effect against colitis in mice, whereas pyroGlu-Asn-Ile-Asp-Pro showed no significant effect. Additionally, administrating pyroGlu-Asn-Ile normalized colitis-induced colonic dysbiosis, whereas pyroGlu-Tyr did not. These results suggest that identified pyroglutamyl peptides exhibited an anti-colitic activity via different mechanisms.

17th September 2016

Dear Professor Rotimi Aluko

I am sending herewith the revised manuscript entitled "**Identification of pyroglutamyl peptides with anti-colitic activity in Japanese rice wine, *sake*, by oral administration in a mouse model**" authored by Kiyono et al., to be considered for publication as a research paper in *Journal of Functional Foods*. Ms. Ref. No.: JFF-D-16-01560R1.

I revised the manuscript in according to reviewers' suggestions.

However, the objective of the present study was to identify anti-colitic peptides by oral administration of realistic dose (< 1 mg/kg body weight). I believe you can understand its difficulty and impact on following studies. The reviewer suggested more detailed characterization of phenotype of disease and molecular mechanism for the peptides. I understand its importance. But as written in the list of changes, these results should be presented in another article, as it requires development of new methods.

I believe we provided sufficient data to complete our mission, identification of active peptide by oral administration. Now, we are examining response to these peptides by in vivo model to identify cells, which respond to the peptides. On the basis of the results, we will elucidate mechanism by *in vitro* assay. All are challenging. Please understand this

step-by-step approach.

We are grateful if you could consider our manuscript for publication in this esteemed journal.

Best regards.

Kenji Sato

Professor of Division of Applied Biosciences

Graduate School of Agriculture, Kyoto University

List of changes

Comments of Reviewer #1

In this manuscript by Kiyono et al., entitled "Identification of pyroglutamyl peptides with anti-colitic activity in Japanese rise wine, sake, by oral administration in a mouse model," Kiyono et al. purified peptides from sake and directly tested them for efficacy in a mouse model of dextran sulfate induced colitis. They observed significant improvement in the clinical score of DSS treated mice by two pyroglutamyl peptides, pyro-Glu-Tyr and polyGlu-Asn-Ile, but not a third pyroGlu-Asn-Ile-Asp-Asn-Pro.

The potential therapeutic application of pyroglutamyl peptides is intriguing and has important clinical relevance to ulcerative colitis patients. Screening the peptides directly in an in vivo mouse model of colitis is a stringent test for efficacy. Significant protective response, particularly for polyGlu-Asn-Ile, is observed. However, a more detailed characterization of the mouse disease phenotype with or without treatment with the peptides would strengthen the manuscript. Specific comments are given below.

Response for reviewer #1 (general).

Thank you for valuable comments. As written in Introduction section, we have concentrated all efforts to identify peptides, which can moderate DSS-induced colitis by ORAL administration of SMALL dose (< 1mg/kg body weight), in Japanese rice wine. It was really challenging. As you also mentioned, identification of active compound by *in vivo* model can provide a stringent result. However, this approach has been hardly used, as it has been really difficult to fractionate food compounds enough amounts for animal experiment. We solved this problem by developing large-scale peptide fractionator (autofocusing). I believe that the identification of anti-colic peptide by oral administration of small dose (< 1mg/kg) has great impact on readers of this journal. To make this point clearer, following sentences were added to end of Introduction section; “The *in vivo* activity-guided fractionation can identify really active peptide by oral administration compared to the conventional *in vitro* activity-guided fractionation without considering bioavailability. However, it has been difficult to fractionate samples to obtain enough amounts of fractions for animal experiment. To solve this problem, the present authors have developed large-scale preparative ampholyte free isoelectric

focusing referred to autofocussing (Hashimoto et al., 2005). By using technique, two anti-colitic peptides by oral administration of small dose (< 1mg/kg body weight) were identified in the present study."

I understand detailed characterization of the mouse disease phenotypes is important and valuable information. However, disease index (DI) and criteria included in DI have been demonstrated to be good marker to evaluate severity of colitis. Then, to complete the present mission, identification of anti-colitic peptide, we believe that DI is enough.

In the present study, we identified two anti-colitic peptides by oral administration of small dose. Now, we are examining response to these peptides by *in vivo* model to identify cells, which respond to the peptides. On the basis of the results, we will elucidate mechanism by in vitro assay. All are challenging. Please understand this step-by-step approach.

To stress importance of this study, following sentences were added to the end of first paragraph of Discussion section; "Therefore, these peptides and food containing these peptides have important clinical relevance to IBD patients. In addition, identification of active peptides allows explore underlying mechanism by using *in vitro* assay system.."

Specific comments by reviewers' #1:

1. There is little characterization of the DSS-induced colitis beyond the clinical score. Histological examination, immune characterization, and/or quantitation of immune cytokines and anti-microbial defensins by qPCR would strengthen the manuscript.

Answer: I agree to this comment. However, in our preliminary experiments, more than 60 peptides were detected in an acid-extract of small intestine, which contain defensins and other antimicrobial peptides. Some peptides shared same sequence, indicating presence of heterogeneity among them possibly due to post-translational modifications. Only some peptides responded to supplementation of the peptides. Then peptidome analysis based two-dimensional LC and mass spectrometry rather than qPCR is now developed. This study can be reported in separate article.

To explain presence of post-translational modifications of antimicrobial peptides, some sentences were added in Discussion section as follows: "In order to identify target cells of these peptides in the body and to understand the underlying mechanism, contents of inflammatory and anti-microbial peptides responding to supplementation of each peptide should be examined. As mammalian antimicrobial peptides are activated by

post-translational modifications (Balducci et al., 2011), a study on the effects of pyroGlu-Tyr, pyroGlu-Asn-Ile, and also pyroGlu-Leu on the proteome of the small intestine and colon of the animals with colitis is under progress."

2. The authors note a difference between the protective effects of the two protective peptides, but no interrogation of the mechanisms of these differences is made. Additional experimental examination as suggested above may provide some insights into the differential effects of the pyroglutamyl peptides. Testing the pyroglutamyl peptides in other experimental colitis models — e.g., other chemical, bacterial, or genetic mouse models — may also add mechanistic insight.
Answer: I agree. Indeed we are examining effect of pyroglutayl peptides on microbiota of different animal model. But it is another study.

3. The peptides are only tested at 2 concentrations (0.1 and 1.0 mg/kg). A more detailed dose response, particularly for pENI, the most effective peptide, would be informative.
Answer: I understand. But this is animal experiment. So many animals should not be used. We provided enough data for identification of the peptides with anti-colitic activity by oral administration of low dose compared to the known compound (written in Discussion).

Minor comments:

1. Editorial corrections would improve the readability of the manuscript, e.g., remove the inclusion of experimental details in the abstract, the use of multiple 'etc' in the introduction, the beginning of the results section without any introduction as to the starting material that is being fractionated. Also, some of the figures could be combined.
Answer: The present in vivo activity-guided fractionation is not commonly used due to technical difficulty (difficult to prepare enough peptide fractions for animal experiment). Then outline of experiments are presented in Abstract. I believe it is important.
Deleted some etc in Introduction section.
Starting material is introduced in the Materials and Methods section.

2. Inconsistency in the data presentation (DAI scores or body weights were variably

reported) led to some confusion and suggestion of data selectivity. In addition, the only significant difference (body weight in mice administered with Fr. III) in Fig. 4 was presented in Supplemental Fig. 2. This data should be move to Fig. 4.
Answer: Supplemental Fig. 1 and 2 were combined to Figure 2 and 4, respectively.

3. The peptides pyroGlu-Tyr, pyroglutamyl-asparaginyl-isoleucine, and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-proline are referred to as pro-Glu-Tyr, polyGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro throughout the text, but pEY, pENI, and pENIDNP in the figures without providing this abbreviation in the text. A consistent nomenclature would make the manuscript easier to read.
Answer: Meaning of the one letter abbreviation was described in Figure legend.

4. The differences in nitric oxide production by LPS-treated RAW cells, though statistically significant, seem rather minimal (Fig. 7). This is the only experiment done with RAW cells and it is presented in one sentence in the results, without rationale or interpretation. Either more context must be given or these results should be removed.
The Figure 7 was deleted.

Reviewer #2: No comments. Very good work, no issues in my opinion.
Thanks!

Highlights

- *Sake* compounds were fractionated by preparative isoelectric focusing and RP-LC.
- Administration of one fraction attenuated DSS-induced colitis in mice.
- Three pyroglutamyl peptides were identified in the active fraction.
- PyroGlu-Tyr and pyroGlu-Asn-Ile attenuated colitis at 0.1–1.0 mg/kg body weight.
- PyroGlu-Asn-Ile normalized colonic dysbiosis in mice with colitis.

Identification of pyroglutamyl peptides with anti-colitic activity in Japanese rice wine, *sake*, by oral administration in a mouse model

Anti-colitic pyroglutamyl peptides in Japanese rice wine

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ABSTRACT

Two pyroglutamyl peptides with anti-colitic activity were identified in Japanese rice wine, *sake*, by oral administration of a small dose (0.1–1.0 mg/kg body weight) and *in vivo* activity-guided fractionation. Compounds in *sake* were fractionated by preparative isoelectric focusing followed by preparative reversed phase-liquid chromatography. Anti-colitic activity was evaluated using a dextran sulfate sodium (DSS)-induced colitis mouse model. The final active fraction contained three pyroglutamyl peptides; pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile) and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-proline (pyroGlu-Asn-Ile-Asp-Asn-Pro). Orally administrating artificially synthesized pyroGlu-Tyr (1.0 mg/kg body weight) and pyroGlu-Asn-Ile (0.1 and 1.0 mg/kg body weight) exhibited a significant protective effect against colitis in mice, whereas pyroGlu-Asn-Ile-Asp-Pro showed no significant effect. Additionally, administrating pyroGlu-Asn-Ile normalized colitis-induced colonic dysbiosis, whereas pyroGlu-Tyr did not. These results suggest that identified pyroglutamyl peptides exhibited an anti-colitic activity via different mechanisms.

Keywords: pyroglutamyl peptide, colitis, *sake*, Japanese rice wine, microbiota.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract and includes ulcerative colitis and Crohn's disease (Podolsky, 2002). Patients suffering from IBD show symptoms characterized by diarrhea, bloody stools, and weight loss. IBD is generally treated with a combination of anti-inflammatory and immunomodulatory drugs. In some cases, these therapies exert limited efficacy and have a risk of adverse effects (Bernstein, 2015). Several efforts have been focused towards the discovery of natural compounds from foods that display protection against IBD. Some plant extracts rich in phytochemicals (Zorrilla et al., 2014; Jia et al., 2014; Cazarin et al., 2015; Zielinska et al., 2015) and some proteins (Lee et al., 2009a, 2009b; Kobayashi et al., 2015) have been demonstrated to moderate IBD in animal models. In those studies, decrease and/or down regulation of pro-inflammatory cytokines and enzymes involved in inflammation such as inducible nitric oxide synthase, cyclooxygenase-2 have been demonstrated. Based on these findings, anti-inflammatory activity in colon has been suggested to be the mechanism for moderation of IBD by such food compounds. The IBD symptoms can be alleviated by pre- and pro-biotics through improvement of colonic microbiota and metabolites of microbiota such as short chain fatty acids (Osman et al., 2008; Damaskos & Kolios, 2008; Hijova & Soltesova, 2013), which can consequently suppress colonic inflammation. Then apparent anti-inflammatory response by ingestion of these foods does not always mean that compounds in the food directly suppress inflammatory action

of host cells in colon. The identification of active compound in the food, which can ameliorate IBD by oral administration, is crucial to elucidate the underlying mechanism of anti-colitic activity. The response of specific cells, including host and exogenous cells, can be elucidated by using compounds isolated from food. It has been demonstrated that food compounds with some beneficial *in vitro* activity such as the anti-oxidant activity, also exert anti-colitic activity (Osman et al., 2008; Larrosa et al., 2009; Yoda et al., 2014). However, in most studies considerably higher doses than normally present in foods were needed to exert beneficial effects against colitis in animal models. Therefore, the beneficial effects of the plant extract could not solely be attributed to the suggested compounds and their *in vitro* activities. Similarly, some amino acids, derived from dietary proteins, also improve the symptoms of IBD; however, the effective doses are higher than the content in the protein. There is a possibility that specific peptides, which could be derived from proteins, might be responsible for suppression of IBD, however, there are few data indicating such active peptide in food. Recently, we found that pyroglutamyl-leucine (pyroGlu-Leu), which was identified in wheat gluten enzymatic hydrolysate as hepatoprotective peptide by oral administration (Sato et al., 2013), also attenuates dextran sulfate sodium (DSS)-induced colitis and normalizes the colonic dysbiosis in mice upon oral administration at 0.1 mg/kg body weight (Wada et al., 2013). Pyroglutamyl peptide, wherein the pyroglutamic acid residue is generated from amino terminus glutaminyl residue, is found in food protein hydrolysates (Sato et al., 1998;

Suzuki et al., 1999) and fermented foods (Kaneko et al., 2011; Kiyono et al., 2013). We previously reported that Japanese rice wine, *sake*, a fermented alcohol beverage made of water and rice inoculated with *Aspergillus oryzae* and *Saccharomyces cerevisiae*, contains 12–15 mg/L of pyroGlu-Leu, and eighteen other short chain pyroglutamyl peptides (Kiyono et al., 2013). In the context of IBD, the objective of the present study was to identify other pyroglutamyl peptides with protective effect against colitis present in *sake* by oral administration in sma ll dose. The *in vivo* activity-guided fractionation can identify really active peptide by oral administration compared to the conventional *in vitro* activity-guided fractionation without considering bioavailability. However, it has been difficult to fractionate samples to obtain enough amounts of fractions for animal experiment. To solve this problem, the present authors have developed large-scale preparative ampholyte free isoelectric focusing referred to autofocussing (Hashimoto et al., 2005). By using technique, two anti-colitic peptides by oral administration of small dose (< 1mg/kg body weight) were identified in the present study.

2. Materials and Methods

2.1. Samples.

Bottled *sake* (15% alcohol content) made from rice polished to 60%, was commercially obtained from Shotoku Brewery (Kyoto, Japan). *Sake* was concentrated (5-fold) by a rotary evaporator at 60°C and used for subsequent experiments.

2.2. Reagents.

Lipopolysaccharide (LPS) from *Escherichia coli*, O111 B4 was obtained from Sigma (Saint Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from JR Scientific (Woodland, CA) and Thermo Fisher Scientific (Waltham, MA), respectively. Dextran sulfate sodium (DSS; average molecular weight, 8,000) was purchased from Seikagaku (Tokyo, Japan). *Pyrococcus furiosus* pyroglutamate aminopeptidase was purchased from Takara Bio (Otsu, Japan). Acetonitrile (HPLC grade), trifluoroacetic acid (TFA), and phenyl isothiocyanate (PITC) were purchased from Wako Chemicals (Osaka, Japan). Triethylamine (TEA) was purchased from Thermo Fisher Scientific. L-pyroglutamic acid was purchased from Nacalai Tesque (Kyoto, Japan). 9-fluorenylmethoxycarbonyl (Fmoc)-Tyr(tBu)-Wang resin, Fmoc-Ile-Wang resin, H-Pro-2-chlorotritylchloride resin, and Fmoc-amino acids for peptide synthesis were purchased from HiPep Laboratories (Kyoto, Japan). Other reagents used were of analytical or higher grade.

2.3. DSS-induced colitis in mice.

Animal experiments were conducted according to a previously described procedure (Wada et al., 2013). Seven-week-old male C57BL/6 mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). The mice (n= 6 or 7) were housed in a room under controlled conditions of 18–24°C, 40–70% relative humidity, and a 12 h light/dark cycle. Mice were allowed free access to food CRF-1 (Oriental Yeast, Tokyo, Japan) and

drinking water during a one-week acclimatization period. All animals were treated and cared for in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, 8th Edition. All experimental procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (M23–37, M24–25, Kyoto, Japan).

Acute colitis was induced by oral administration of 2.5 or 3.0% (w/v) DSS dissolved in drinking water for 7 days. The working concentration of DSS (2.5% or 3.0%) was determined by preliminary experiments by using the same batch of animals to induce an identical grade of inflammation (more than disease activity index score 2) as described in a previous study (Wada et al., 2013). Mice were allowed free access to drinking water either containing DSS (DSS +) or without DSS (DSS –). Samples were dissolved in distilled water and administered by gavage in aliquots of 200 μL once a day during the entire induction period. The vehicle group of mice received an identical amount of distilled water. The body weight of each mouse was measured on days 0, 3, 5, and 7. Mice were sacrificed on day 7 and the entire colon was removed. Immediately after colon resection, the colon length was measured.

For the identification of active fraction, the severity of colitis was evaluated by a disease activity index (DAI) score as previously reported (Wada et al., 2013). The DAI score was determined by scoring of weight loss, stool consistency and bleeding as described previously (Table 1) (Murano et al., 2000; Naito et al., 2006).

2.4. Fractionation of compounds in *sake* by preparative isoelectric focusing.

For animal experiments, the concentrated compounds in *sake* were fractionated by preparative isoelectric focusing based on the amphoteric nature of the sample according to a previously described method (Hashimoto et al., 2005) with slight modifications (Sato et al., 2013). This method has been referred to as autofocusing. An autofocusing apparatus with 12 compartments (75 mm in length × 80 mm in width × 85 mm in height for each compartment), separated by a thin agarose gel layer was prepared. Phosphoric acid at 0.03 M concentration and 0.1 M sodium hydroxide were loaded in the compartments at either ends of the tank, which were used as anode and cathode, respectively. Ten compartments in the center of the tank were used as sample compartments and were numbered from the anode side (No. 1) to the cathode side (No. 10). Sample compartments number 8 and 9 were each filled with 500 mL of the 5-fold *sake* concentrate. The other sample compartments were filled with deionized water. Autofocusing was performed at constant voltage mode at 500 V for 24 h. Following this, all fractions were collected and their pH adjusted to 7.0 by addition of 1 M HCl or sodium hydroxide, and used for subsequent experiments.

2.5. Fractionation of compounds by preparative reversed phase-liquid chromatography.

The fraction obtained after autofocusing, which attenuated DSS-induced colitis in mice, was subjected to a second fractionation step using preparative reversed phase-liquid

chromatography (RP-LC). The RP-LC system consisted of a column (160 × 15 mm, Millipore, Billerica, MA) packed with YFLC Gel ODS (50 μm particle size, 120 Å pore size, Yamazen, Osaka, Japan), a pump (FL600A, Yamazen), a gradient controller (Gradicon III AC-5900, Atto, Tokyo, Japan), and a gradient mixer (AC-5905, Atto). The column was equilibrated with 10 mM HCl at a flow rate of 10 mL/min. Following this, 80 mL of the autofocusing fraction, diluted to 3.0 mg peptide/mL for animal experiments, was loaded to the column using the pump. Elution was performed using a binary linear gradient consisting of 10 mM HCl (solvent A) and 10 mM HCl containing 60% (v/v) acetonitrile (solvent B). Gradient profile was 0% B for 1 min followed by 0–100% B over 20 min and 100% B for 2 min. Fractions were collected every 0.5 min. Elution was monitored by absorbance at 214, 230, and 254 nm using UV/Vis spectrometer (DU640, Beckman Coulter, Brea, CA).

2.6. Identification of pyroglutamyl peptides.

Identification of pyroglutamyl peptides was performed by using methods described previously (Kiyono et al, 2013) with slight modifications. About 4 mL aliquots of the preparative RP-LC fraction, which attenuated DSS-induced colitis in mice, was dried under vacuum and dissolved in 400 μL of 0.1% (v/v) TFA containing 30% acetonitrile. This sample was clarified by passing through a spin column (15 × 7 mm i.d., 5 μm pore size, Ultrafree-MC, Millipore) packed with Sephadex G-25 fine grade (GE Healthcare, Buckinghamshire, UK), which was pre-equilibrated with the same solvent. Sample was

eluted from the spin column by centrifugation at 7,000 rpm for 1 min after which the spin column was washed with 50 µL of the same solvent, and the effluents were combined (450 µL of total volume). A 200-µL aliquot of the clarified sample was loaded onto a size exclusion chromatography (SEC) column (Superdex Peptide 10/30 GL, GE Healthcare), pre-equilibrated with 0.1% TFA containing 30% acetonitrile at a flow rate of 0.5 mL/min. Fractions (21–45 in number) were collected at 1 min interval for 20–45 min. This procedure was performed in duplicate.

The SEC fractions 31–45 above were dried under vacuum, dissolved in 200 µL of distilled water, and subjected to solid phase extraction using a strong cation exchange resin (AG50W-x8, hydrogen form, 100–200 mesh, Bio-Rad Laboratories, Hercules, CA) to separate the pyroglutamyl peptides from peptides bearing amino groups. The sample (200 µL) was loaded onto the spin column packed with the resin, which was pre-equilibrated with 10 mM HCl containing 50% (v/v) methanol, and eluted by centrifugation at 5,000 x g for 1 min. The columns were washed twice with 100 µL of 50% methanol. Unabsorbed effluents were combined and dried under vacuum, and then dissolved in 200 µL of distilled water. Unabsorbed samples (80 µL) were injected to a reversed phase-high performance liquid chromatography (RP-HPLC) column (Inertsil ODS-3, 250 × 4.6 mm, 5 µm, GL Science, Tokyo, Japan) pre-equilibrated with 0.1% formic acid at a flow rate of 1.0 mL/min. Elution was performed using a binary linear gradient consisting of 0.1% formic acid (solvent A) and 0.1% formic acid containing 80%

acetonitrile (solvent B). The gradient profile was as follows: 0–30% B from 0–30 min; 30–100% B from 30–35 min; 100% B from 35–40 min; 100–0% B from 40–40.1 min; 0% B from 40.1–50 min. Column temperature was maintained at 45°C. Elution was monitored at 214 nm and peaks were collected. Two sets of sample aliquots (100 µL) were transferred to 1.5-mL centrifuge tubes and dried under vacuum. One set was used for pyroglutamate aminopeptidase digestion and another set was used as blank. One hundred microliter of 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol and 1 mM ethylendiaminetetraacetic acid was added to the blank tubes. Eighty microliter of the same buffer and 20 µL of pyroglutamate aminopeptidase solution (0.4 mU/20 µL of the same buffer) were added to the tubes used for the digestion. The enzymatic reaction was carried out at 60°C for 1 h. The reaction was terminated by drying under vacuum. The amino groups liberated upon digestion from the pyroglutamyl peptide underwent a reaction with PITC and the resulting phenyl thiocarbamyl (PTC) derivatives were resolved by RP-HPLC as previously described (Kiyono et al., 2013). Peaks appearing exclusively in the enzyme digests were collected and dried under vacuum, following which a ‘re-drying solution’ (20 µL) consisting of methanol, water, and TEA (7:1:2) was added and the contents were re-dried under vacuum to remove ammonia. The residual contents were dissolved in 30% methanol and applied to an automatic peptide sequencer operating based on Edman degradation (PPSQ-21, Shimadzu, Kyoto, Japan). The peptide sequencer was reprogrammed to begin with TFA cleavage reaction.

Aliquots of the RP-HPLC fractions without pyroglutamate aminopeptidase treatment were subjected to direct infusion electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis using 3200 QTRAP System (AB SCIEX, Framingham, MA).

2.7. Synthesis of pyroglutamyl peptides.

Pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile), and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-proline (pyroGlu-Asn-Ile-Asp-Asn-Pro) were synthesized by Fmoc solid phase synthesis using an automatic peptide synthesizer (PSSM-8, Shimadzu) according to the supplier's protocols. The synthesized pyroglutamyl peptides were cleaved from the resin using TFA and dried under vacuum. These peptides were then purified using RP-HPLC (Cosmosil 5C_{18}-MS-II, 250 × 10 mm, 5 μm, Nacalai Tesque) using an acetonitrile/10 mM HCl solvent system.

2.8. Amino acid analysis.

Peptide content was determined by amino acid analysis after HCl hydrolysis. For this purpose, samples were hydrolyzed using 6 M HCl vapor at 150°C as described previously (Bidlingmeyer, Cohen & Tarvin, 1984). The amino acids were reacted with PITC and the resulting PTC amino acids were resolved using the same conditions used for isolation of PTC-peptides in pyroglutamate aminopeptidase digest as described previously (Kiyono et al., 2013).

2.9. Sugar analysis.

Samples were diluted with distilled water to result in absorbance values within the standard curve range. Concentrated sulfuric acid (150 μL) and 10% (w/v) phenol (15 μL) were added to samples (30 μL) in 1.5 mL centrifuge tubes and incubated for 10 min. The tubes were then vortexed and incubated further for 30 min. Reaction mixtures were transferred to a 96 well plate and absorbance was monitored at 490 nm. The sugar contents of samples are presented as glucose equivalents.

2.10. Detection of pyroGlu-Leu.

PyroGlu-Leu in preparative RP-LC fractions was detected by LC-MS/MS analysis as described previously (Wada et al., 2013).

2.11. Microbiota analysis.

DNA was extracted from mice feces by using a QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands). Analysis of fecal bacterial flora in the extracted DNA was outsourced to Primary Cell, Division of Cosmo Bio (Sapporo, Japan). The population of phylum *Bacteroidetes* and *Firmicutes* in mice feces were evaluated as the ratio of each phylum to all bacteria by genomic DNA coding 16S rRNA. The DNA amplification was carried out by real-time polymerase chain reaction (RT-PCR). HDA1-GC (5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACG GGAGGCAGCAGT3') and HDA2 (5'GTATTACCGCGGCTGCTGGCAC3') were used for detection of all bacteria (Tannock et al., 2000). Bact934F (5'GGARCATGTGGTTTAATTCGATGAT3') and Bact1060R

(5'AGCTGACGACAACCATGCAG3') were used for detection of *Bacteroidetes*. Firm934F (5'GGAGYATGTGGTTTAATTCGAAGCA3') and Firm1060R (5'AGCTGACGACAACCATGCAC3') were used for detection of *Firmicutes* (Guo et al., 2008). Real-time PCR was performed using a LightCycler 480 (Roche Applied Science, Mannheim, Germany) according to the SYBR Green I Master Protocol.

2.12. Statistical analysis.

The body weight change, colon length, and DAI scores in DSS-induced colitis mice are presented as mean ± standard error. The ratio of two microorganisms (*Firmicutes*/*Bacteroidetes*) in the feces of mice is also presented as mean ± standard error. Data were subjected to one-way ANOVA with Dunnett's multiple comparison of means test. Differences of $p < 0.05$ were considered significant. Statistical analysis was performed using Ekuseru-Toukei 2010 Version1.11 (Social Survey Research Information, Tokyo, Japan).

3. Results

3.1. Fractionation by autofocusing.

The pH of the ten fractions (Fr.) produced by autofocusing of the *sake* sample was about 3.0 between Fr. 1–8 then rose sharply in Fr. 9 and 10 to pH 9.0 and 13.0, respectively (Figure 1A). Approximately 80% of total peptides were recovered in Fr. 8 and 9 (Figure 1B). Only negligible amounts of peptides were recovered in Fr. 1 and 2.

Peptides present in Fr. 3–8 consisted of higher levels of acidic amino acids (such as glutamic acid) after HCl hydrolysis, and peptides in Fr. 10 consisted of higher levels of basic amino acids (such as arginine and lysine) compared with the other fractions (Figure 1C). This charge-based separation of amino acids indicated successful fractionation of peptides contained in *sake* based on their isoelectric points. A relatively high sugar content (~ 24%) was recovered in the acidic fractions, particularly Fr. 6 and 7 (Figure 1D). All autofocusing fractions were mixed with ethanol at end concentrations of 75% (v/v) in order to remove agarose, which was derived from the separator of autofocusing. Based on the results of autofocusing, Fr. 1–5 and 6–7 obtained after autofocusing were pooled into two different fractions and used further for animal experiments. Fr. 8, 9, and 10 were used for animal experiments without pooling. The samples were further concentrated by a rotary evaporator to remove ethanol and then diluted with distilled water to give 3.0 mg/mL peptide (total amino acids) and administered to the mice with DSS-induced colitis at 30 mg/kg body weight a day.

The induction of colitis was confirmed by a significant increase in DAI score observed after treatment with DSS (DSS +), as compared to the mice that did not receive DSS (DSS -) (Figure 2A). Only the mice receiving autofocusing Fr. 8 showed a significant decrease in DAI score compared to the vehicle group (DSS +). However, the crude *sake* concentrate, before undergoing autofocusing, exerted no significant decrease in the DAI score. The mice receiving Fr. 6–7, 8, and 9 significantly attenuate body weight loss

(Figure 2B), and there was no significant difference in colon length among all groups (Figure 2C). Based on the above data, Fr. 8 was selected for a further fractionation treatment to identify active compounds.

3.2. Fractionation by RP-LC.

Fraction 8 (3.0 mg/mL peptide) was subjected to further fractionation by RP-LC. Following the elution of non-absorbed compounds from the RP-LC column, the absorbed compounds were eluted by increasing acetonitrile concentrations (Figure 3A). Free amino acids and sugars were mostly eluted in the non-absorbed fraction (Figure 3A and B). The peptide content could be estimated by subtracting free amino acids from total amino acids in HCl hydrolysate. Peptides were eluted in both the non-absorbed and the absorbed fractions. A strong UV absorbance peak was observed at 20–21 min (Figure 3C). Thus, four fractions were collected and labeled as follows: Fr. I (0–14 min), Fr. II (14–17.5 min), Fr. III (17.5–20 min) and Fr. IV (20–31 min). These fractions were freeze-dried to remove acetonitrile and dissolved into 80 mL of distilled water, which were further used for animal experiments.

Although statically insignificant ($p > 0.05$), the smallest DAI score was observed in RP-LC Fr. III group ($p = 0.08$) among the mice receiving Fr. I-IV compared to the vehicle group (DSS +) (Figure 4A). In addition, administration of Fr. III significantly averted body weight loss compared to the vehicle group (DSS +) (Figure 4B), and there was no

significant difference in colon length among all groups (Figure 4C). Based on these results, Fr. III was used for further experiments.

3.3. Identification of pyroglutamyl peptides with protective effect against DSS-induced colitis in RP-LC Fr. III.

Peptides contained in Fr. III were initially resolved by SEC. Approximately 75% of the total peptides eluted between 30–36 min corresponding to Fr. 31–36 (data not shown). Pyroglutamyl peptides in Fr. 31–36 were separated from peptides bearing amino groups by solid phase extraction and then resolved by RP-HPLC. The major peaks obtained after RP-HPLC; marked as a, b, and c; were collected (Figure 5A) and. aliquots of the collected fractions were digested with pyroglutamate aminopeptidase. The digested and non-digested samples were allowed to react with PITC and the resulting PTC derivatives were resolved by a second RP-HPLC separation. Peaks a', b', and c' appeared only in case of enzyme digests (Figure 5B). These PTC derivatives were identified by Edman degradation as follows; peak a', tyrosine; peak b', asparaginyl-isoleucine and peak c', asparaginyl-isoleucyl-asparagyl-asparaginyl-proline. This fact indicated that peaks a, b, and c in Figure 5A consisted of pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile), and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-

proline (pyroGlu-Asn-Ile-Asp-Asn-Pro), respectively. ESI-MS and MS/MS analyses of peaks a–c also confirmed the presence of pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro, respectively (Supplemental Fig 1–3).

Three identified pyroglutamyl peptides were chemically synthesized and administered to the mice with DSS-induced colitis. Although statistically insignificant, ($p > 0.05$), the administration of these pyroglutamyl peptides showed a tendency towards reduced body weight loss compared to the vehicle group (DSS +) (Figure 6A). The pyroGlu-Asn-Ile group (1.0 mg/kg body weight) showed the smallest body weight loss. Administration of pyroGlu-Tyr and pyroGlu-Asn-Ile, at 1.0 mg/kg body weight, significantly reduced colon shortening ($p < 0.05$ and $p < 0.01$, respectively) (Figure 6B). Additionally, pyroGlu-Asn-Ile significantly decreased DAI score compared to the vehicle group (DSS +) in a dose dependent manner (Figure 6C). However, pyroGlu-Asn-Ile-Asp-Asn-Pro did not significantly attenuate colon shortening and DAI score increasing of DSS-induced colitis mice.

3.4. Effects of pyroglutamyl peptides on colonic microbiota.

Without administration of pyroglutamyl peptides, DSS treatment resulted in an increase in the population of colonic *Firmicutes* and decrese in that of *Bacteroidetes*. Thus, a significantly increased ratio between the colonic populations of *Firmicutes* to *Bacteroidetes* by DSS treatment was observed (Figure 6D). The ratio significantly decreased similar to the level of the normal mice (DSS –) upon administration of

pyroGlu-Asn-Ile. On the other hand, pyroGlu-Tyr did not significantly affect the ratio of *Firmicutes* to *Bacteroidetes*.

4. Discussion

It is known that some crude plant extracts have been demonstrated to have anti-colitic activity in animal models (Zorrilla et al., 2014; Jia et al., 2014; Cazarin et al., 2015; Zielinska et al., 2015). Some compounds with demonstrated *in vitro* anti-oxidant, anti-inflammatory and other similar beneficial activities have been selected as candidate anti-colitic compounds. However, relatively large dose of these compounds are necessary for improving colitis in animal model possibly due to low bioavailability and rapid metabolism of these compounds (Larrosa et al., 2009; Shigeshiro, et al., 2013; Sun et al., 2016). To address this problem, we used *in vivo* activity-guided fractionation rather than *in vitro* activity-guided fractionation for identification of anti-colitic peptides in *sake*. The present *in vivo* activity-guided fractionation revealed that pyroGlu-Tyr and pyroGlu-Asn-Ile contained in *sake* attenuated DSS-induced colitis in mice upon oral administration in relatively low concentrations (1.0 mg/kg body weight for pyroGlu-Tyr, and 0.1 and 1.0 mg/kg body weight for pyroGlu-Asn-Ile). This protective effect exerted by the pyroglutamyl peptides was better at lower concentrations when compared to the dose dependence displayed by other natural compounds reported previously. Therefore, these peptides and food containing these peptides have important clinical relevance to

IBD patients. In addition, identification of active peptides allows explore underlying mechanism by using *in vitro* assay system.

A previous study reported that pyroGlu-Leu, the major pyroglutamyl peptide in *sake* (12–15 mg/L) (Kiyono et al., 2013) can attenuate DSS-induced colitis upon administration at 0.1 mg/kg body weight, while doses higher than 0.5 mg/kg body weight and lower than 0.01 mg/kg body weight do not exert any protective effects in the same animal model (Wada et al., 2013). The preset *in vivo* activity-guided fractionation did not detect pyroGlu-Leu as active compound. LC-MS/MS analysis revealed that pyroGlu-Leu was recovered in RP-LC Fr. IV (data not shown). However, as shown in Figure 4, DAI score of the mice receiving RP-LC Fr. IV was higher than that of the mice receiving RP-LC Fr. III possibly due to the higher pyroGlu-Leu content (0.31 mg/kg body weight) than optimized dosage (Wada et al., 2013).

A previous study demonstrated that in comparison to the mice without colitis, DSS-mediated colitis induced mice showed a higher ratio of *Firmicutes* to *Bacteroidetes*, the two major phyla of mice colonic microbiota, and administration of pyroGlu-Leu in mice with colitis normalized their colonic dysbiosis (Wada et al, 2013). This study confirms the previous finding related to changes in colonic microbiota in mice with DSS-induced colitis. Additionally, this study revealed that administration of pyroGlu-Asn-Ile also normalized the colonic dysbiosis in DSS-induced colitis mice, while administration of pyroGlu-Tyr did not affect the colonic microbiota. In contrast,

colonic myeloperoxidase activity was decreased by administration of pyroGlu-Tyr compared to that of the vehicle group (DSS +), indicating that colonic inflammation was suppressed by administration of pyroGlu-Tyr (Supplemental Fig. 4). These results indicate that attenuation of colitis does not always result in normalization of colonic microbiota. However, it has been indicated that intestinal dysbiosis plays significant role in inflammatory bowel disease (Sartor & Mazmanian, 2012). Therefore, normalization of colonic dysbiosis by administration of pyroGlu-Asn-Ile can potentially attenuate colitis.

In addition, to pyroGlu-Leu, pyroGlu-Tyr and pyroGlu-Asn-Ile have potential anti-inflammatory activity (Figure 7), however, the target cells responding to each pyroglutamyl peptide remains to be solved. In order to identify target cells of these peptides in the body and to understand the underlying mechanism, contents of inflammatory and anti-microbial peptides responding to supplementation of each peptide should be examined. As mammalian antimicrobial peptides are activated by post-translational modifications (Balducci et al., 2011), a study on the effects of pyroGlu-Tyr, pyroGlu-Asn-Ile, and also pyroGlu-Leu on the proteome of the small intestine and colon of the animals with colitis is under progress.

The present study clearly demonstrates that *sake* contains at least three pyroglutamyl peptides (pyroGlu-Leu, pyroGly-Tyr, and pyroGlu-Asn-Ile), which can attenuate DSS-induced colitis in mice at doses of 0.1-1.0 mg/kg body weight. However, crude *sake* did not show anti-colitic activity, rather it has been demonstrated that some organic acids

such as lactic acid and succinic acid, which are abundantly present in *sake* (Tadenuma, 1966), may induce diarrhea and worsen the symptom of colitis (Tsukahara & Ushida, 2002). Thus *sake* contains both components with beneficial and adverse effects on colitis and optimizing the contents of these compounds could produce functional beverages against colitis. Currently, studies on producing anti-colitic beverages by optimizing fermentation conditions for *sake* are in progress.

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Figure captions

Figure 1. Fractionation of *sake* concentrate by ampholyte-free preparative isoelectric focusing (autofocusing). pH value (A), total peptides content (total constituent amino acids after HCl hydrolysis) (B), ratio of the basic, neutral, and acidic amino acids to the total peptide contents (C), and sugar content as glucose equivalent (D) of each autofocusing fraction are shown.

Figure 2. Effect of administrating of autofocusing fractions of *sake* on DAI scores (A), body weight change (B), and colon length (C) in mice with DSS-induced colitis. Values are presented as mean ± standard error. DSS – and DSS + represents the mice, which were allowed free access to DSS free drinking water and water containing DSS, respectively. Sample doses were adjusted to 30 mg /kg body weight for peptide (total amino acids)/content. ** represents $p < 0.01$, when compared to the vehicle group (DSS +) by Dunnett's test.

Figure 3. Re-fractionation of compounds in autofocusing Fr. 8 by preparative RP-LC. Arrows indicate initiation of an acetonitrile gradient. Free amino acids content (white circle) and total constituent amino acids content obtained after HCl hydrolysis (black circle) (A), sugar content as glucose equivalent (B), and absorbance in the UV range (C) are shown.

Figure 4. Effect of preparative reversed-phase liquid chromatography (RP-LC) Fr. I–IV on DAI scores (A), body weight change (B), and colon length (C) in mice with DSS-induced colitis. DSS – and DSS + represents the mice that were allowed free access to normal water and water containing DSS, respectively. Values are presented as mean ± standard error. ** represents $p < 0.01$, when compared to the vehicle group (DSS +) by Dunnett's test.

Figure 5. Identification of pyroglutamyl peptides in RP-LC Fr. III. Size exclusion chromatography (SEC) Fr. 31–36 of the RP-LC Fr. III were further subjected to RP-HPLC (A). Compounds in peaks a, b, and c in panel A were digested with pyroglutamate aminopeptidase and derivatized with phenyl isothiocyanate. The resultant phenyl thiocarbamyl-amino acids/peptides were resolved by second RP-HPLC (B). +, sample digested with pyroglutamate aminopeptidase; −, non-digested samples. Peaks marked a', b', and c' in panel B yielded phenyl thiohydantoin-amino acids/peptides upon Edman degradation. Peaks marked with asterisks did not liberate amino acids or peptides after pyroglutamate aminopeptidase digestion (A) and Edman degradation (B).

Figure 6. Effects of pyroGlu-Tyr (pEY), pyroGlu-Asn-Ile (pENI), and pyroGlu-Asn-Ile-Asp-Asn-Pro (pENIDNP) on DSS-induced colitis in mice. Body weight

change (A), colon length (B), DAI score (C), and ratio of the colonic population of *Firmicutes* to *Bacteroidetes* (D) are shown. DSS – and DSS + represents the mice that were allowed free access to normal drinking water and water containing DSS, respectively. V represents the vehicle group. Values are presented as mean ± standard error. * and ** represent $p < 0.05$, and < 0.01, respectively, when compared to the vehicle group (DSS +) by Dunnett's test.

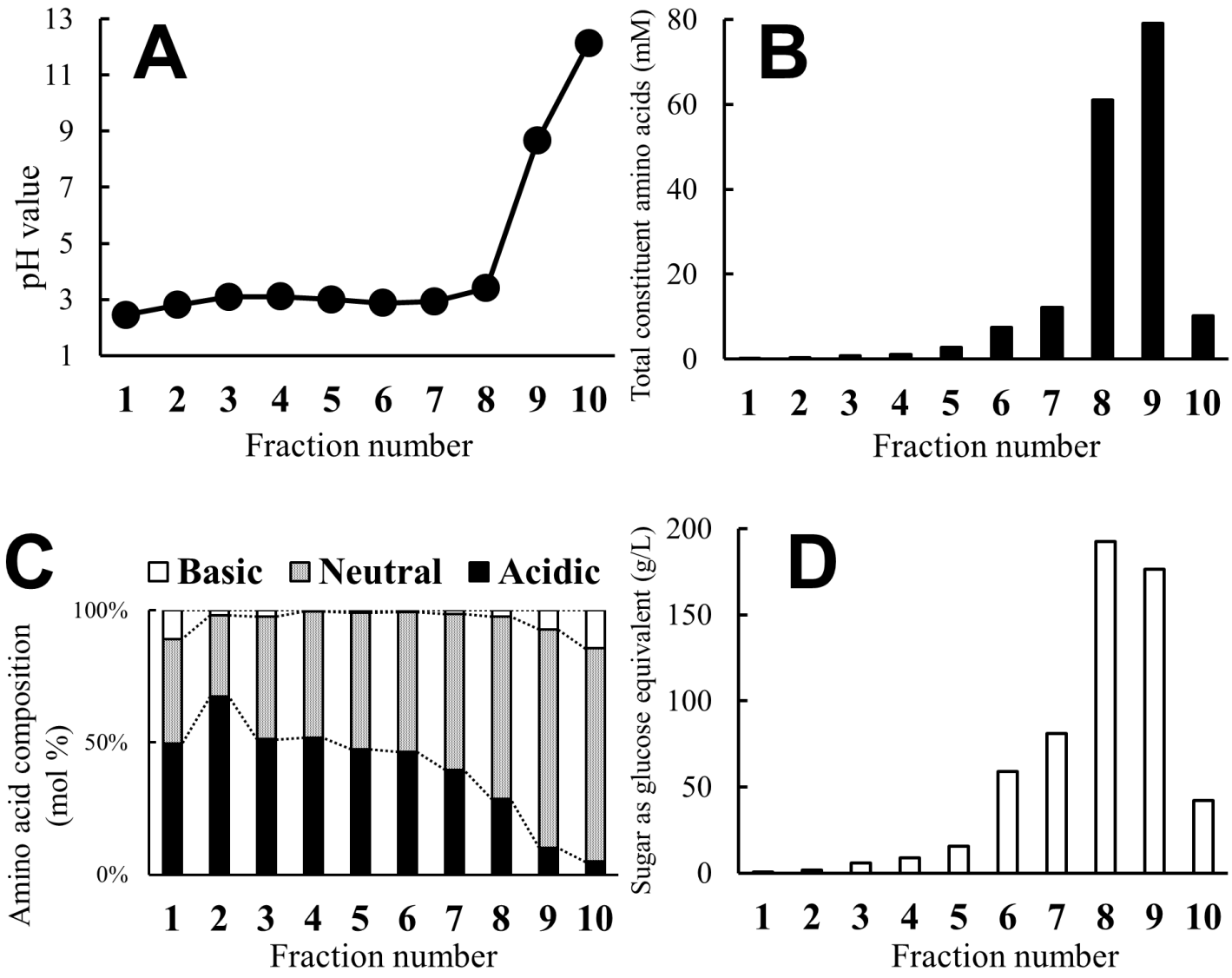
Table 1. Scoring system of disease activity index (DAI)*

Scoring	Weight loss (%)	Stool** consistency	Stool bleeding
0	None	Normal	Negative
1	1–5	Mild loose	Light
2	5–10	Loose	Mild
3	10–20	Mild bleeding	Severe
4	>20	diarrhea	Entire

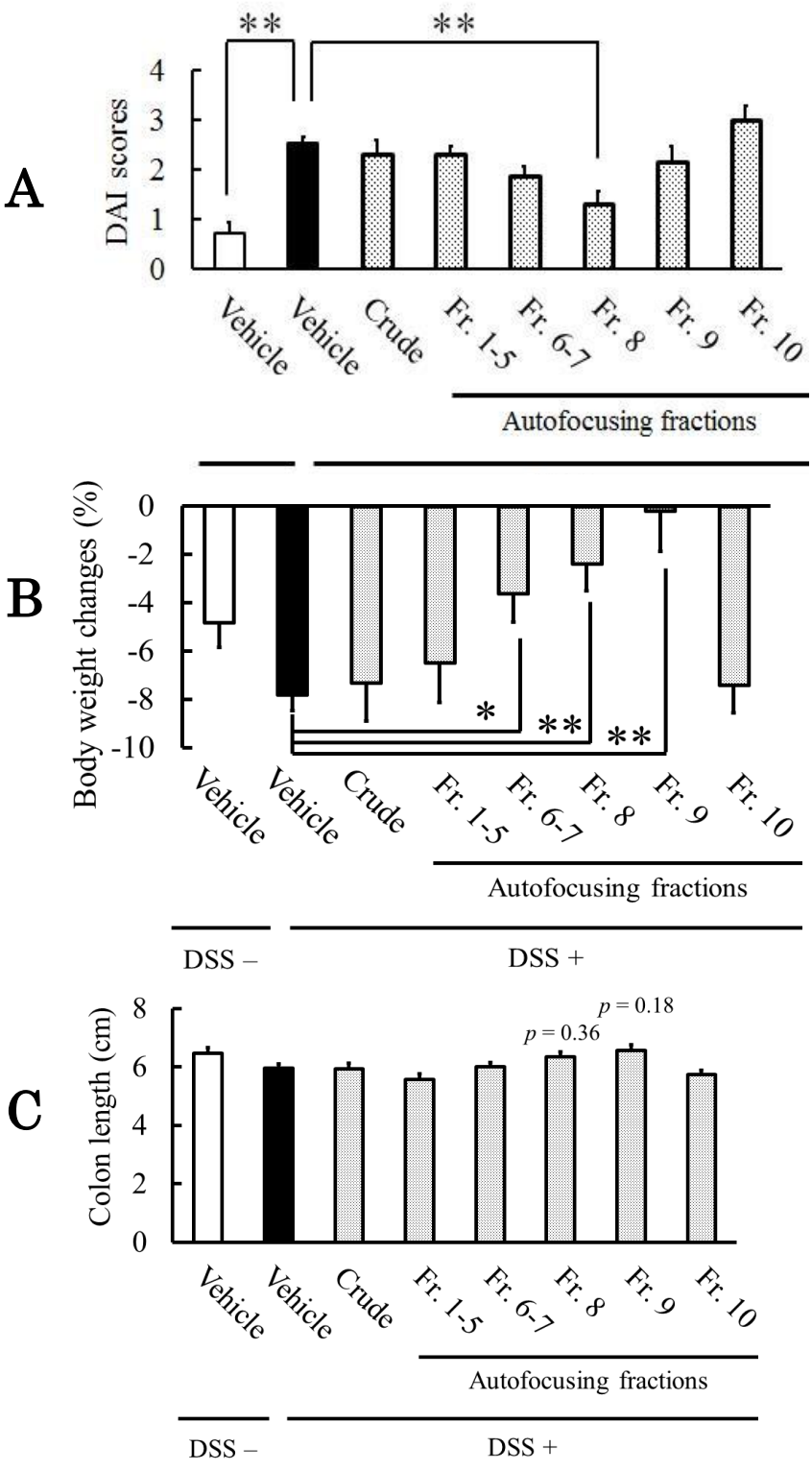
*DAI = (Combined scores of weight loss, stool consistency and bleeding)/3.

**Normal, formed pellets; Loose, pasty stools that do not stick to the anus; diarrhea, liquid stools that stick to the anus.

Download Figure: Kiyono Fig. 1.docx



Kiyono et al., Fig. 1



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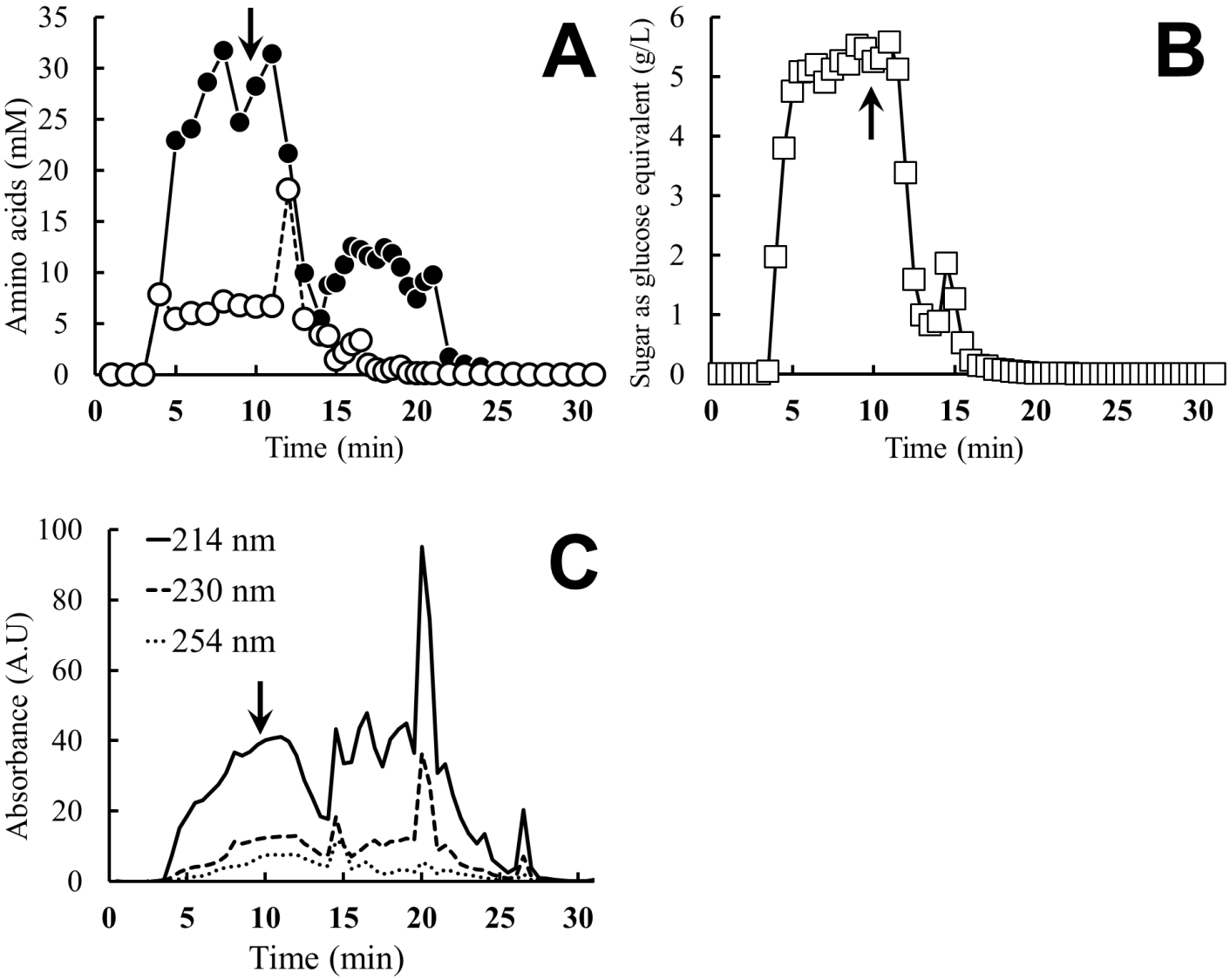

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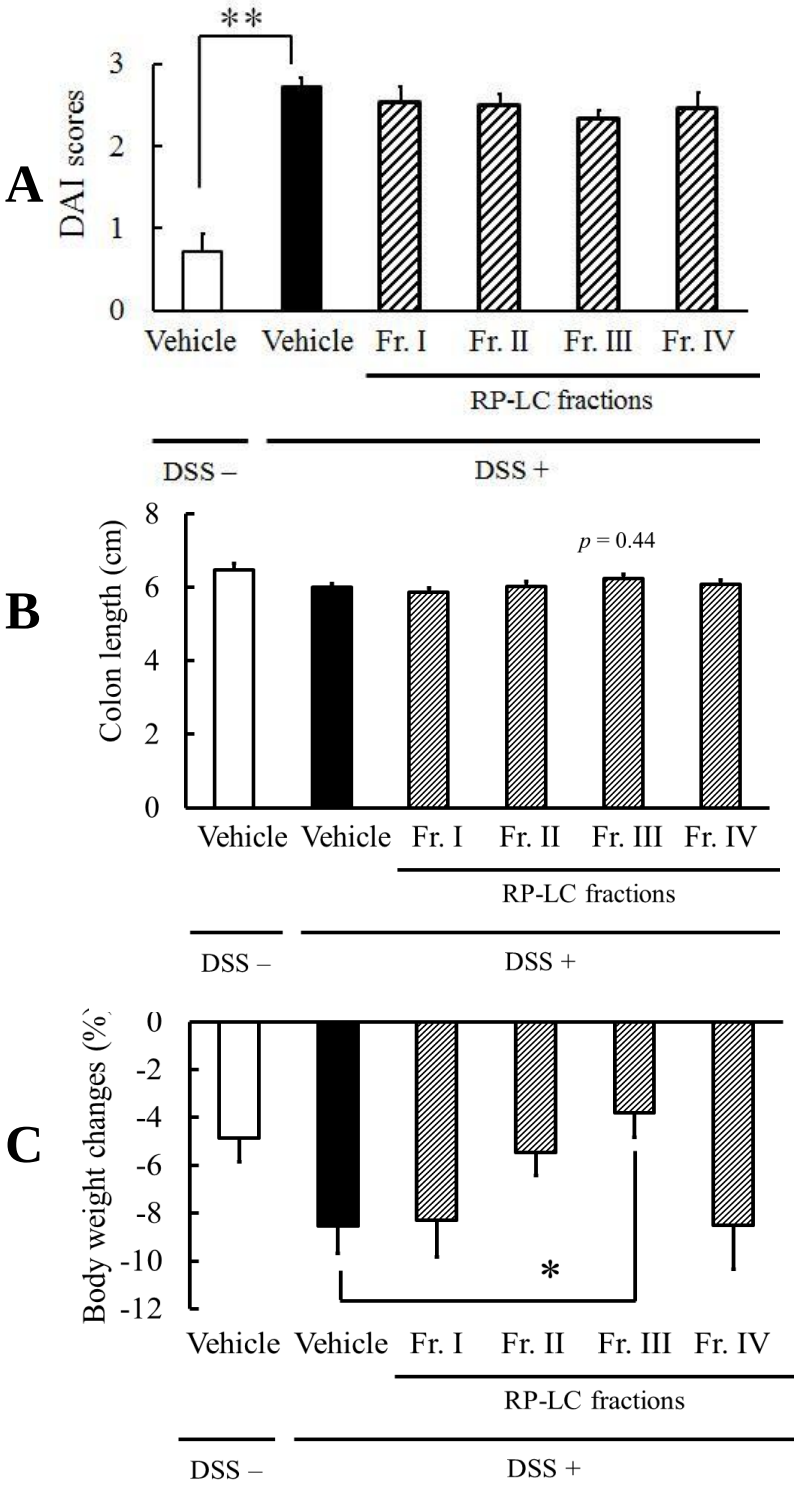

Figure 4. Kiyono et al.

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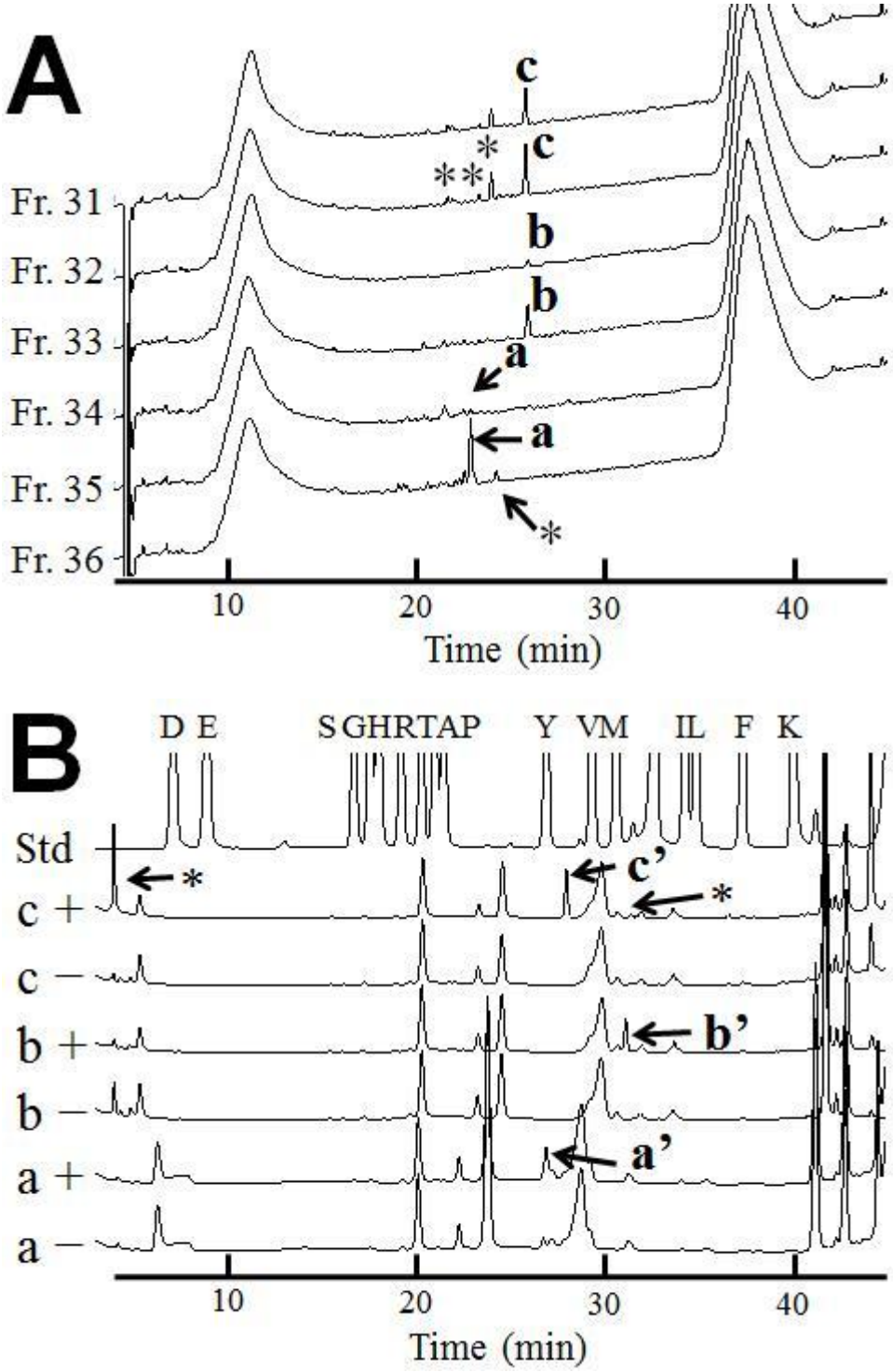

Kiyono et al., Fig. 5

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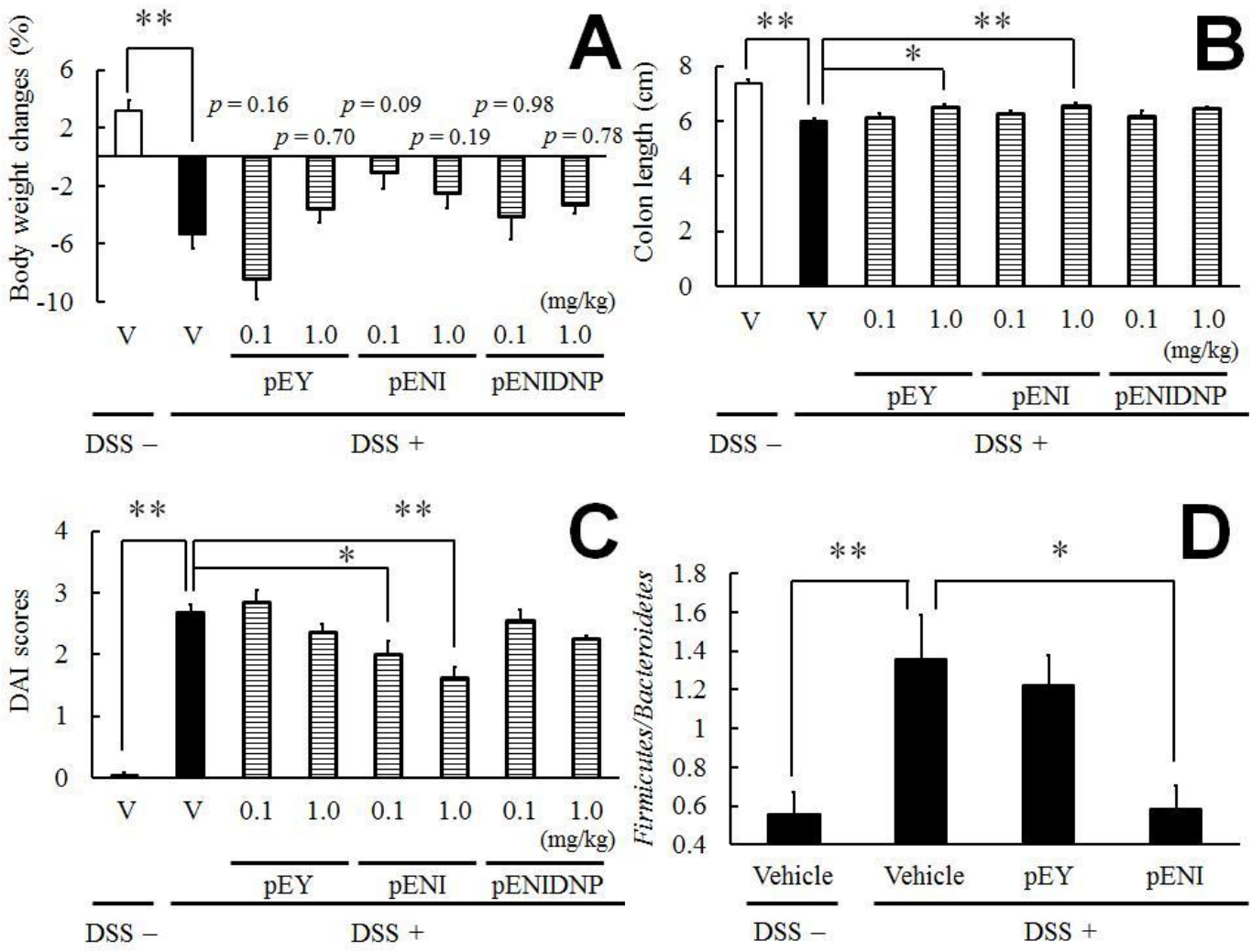
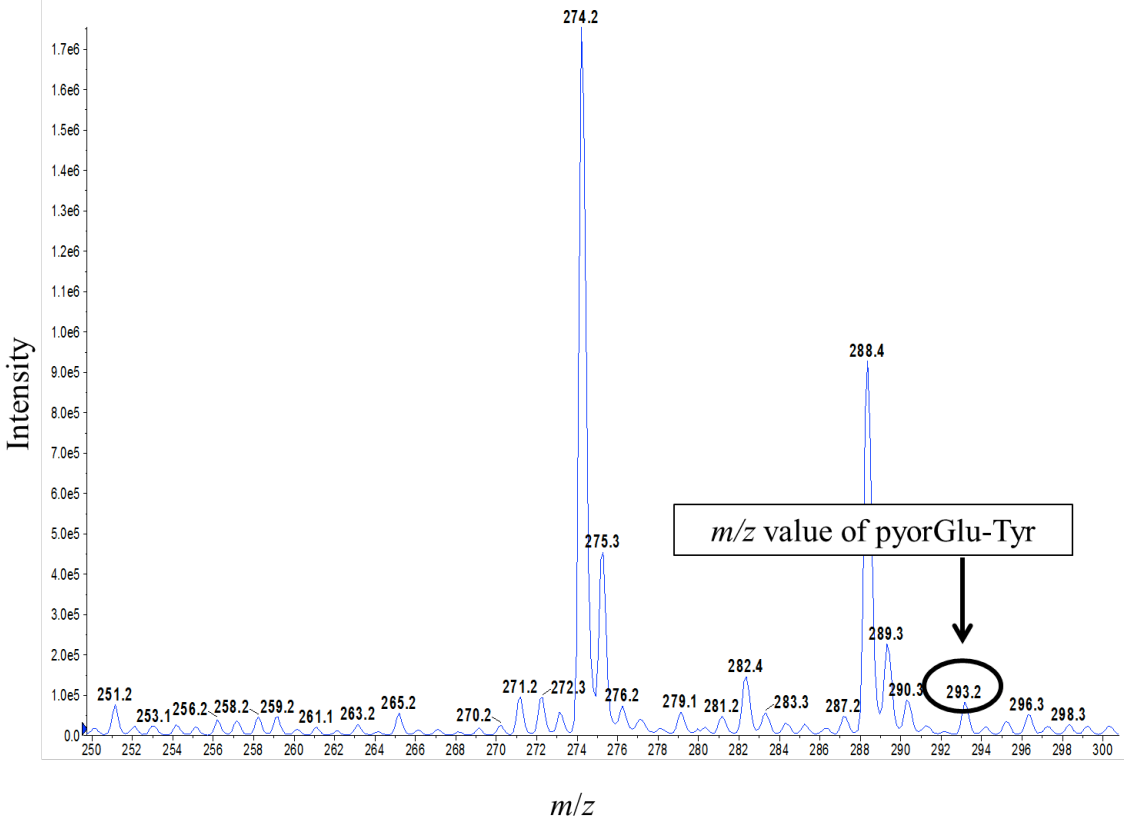
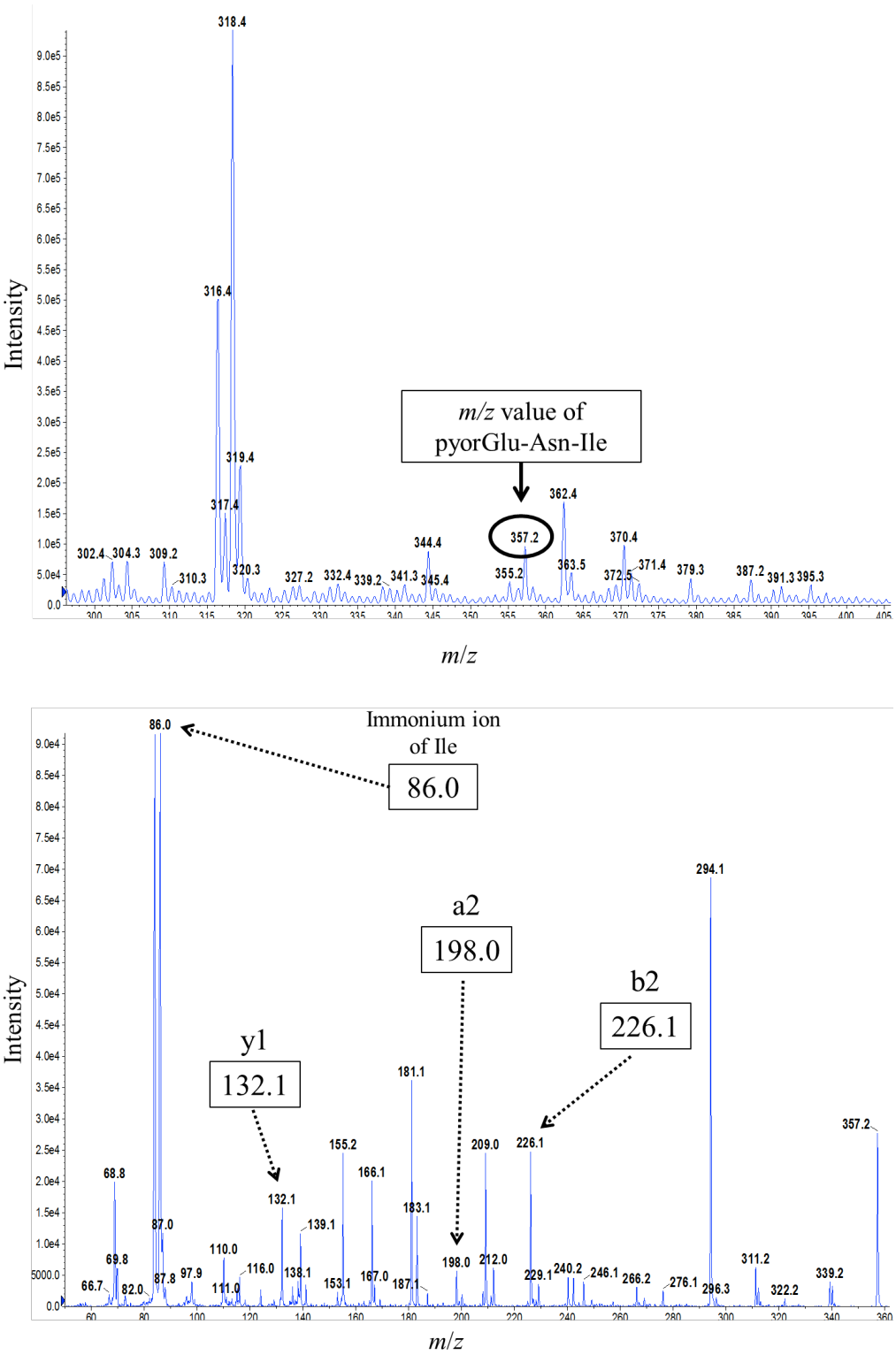


Kiyono et al., Fig. 6

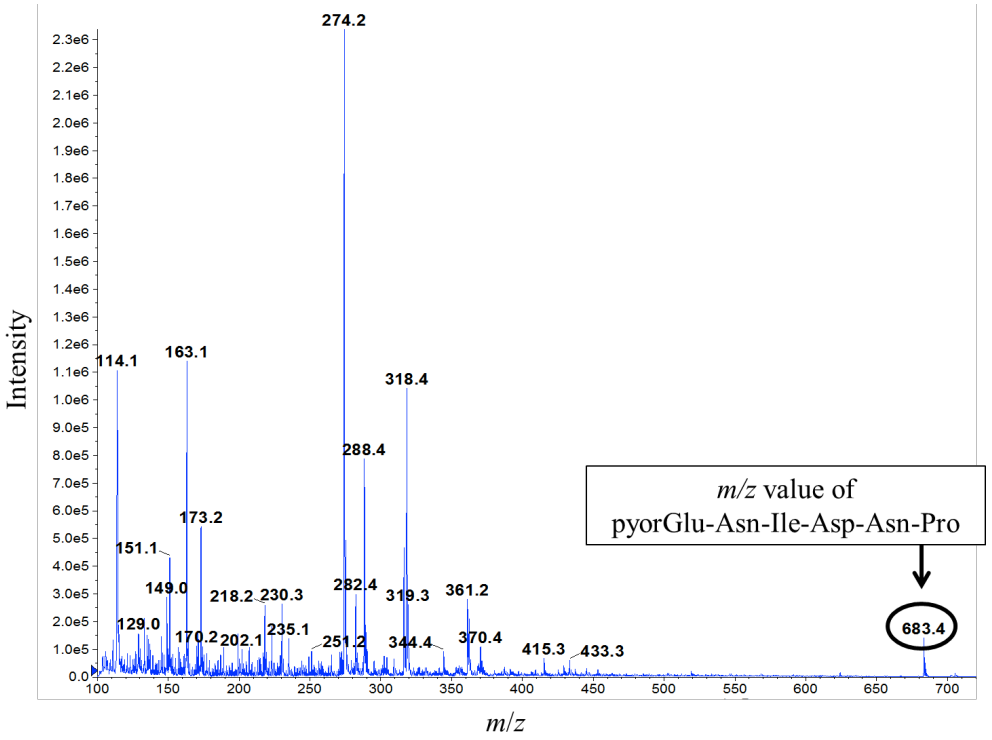
Supplementary file

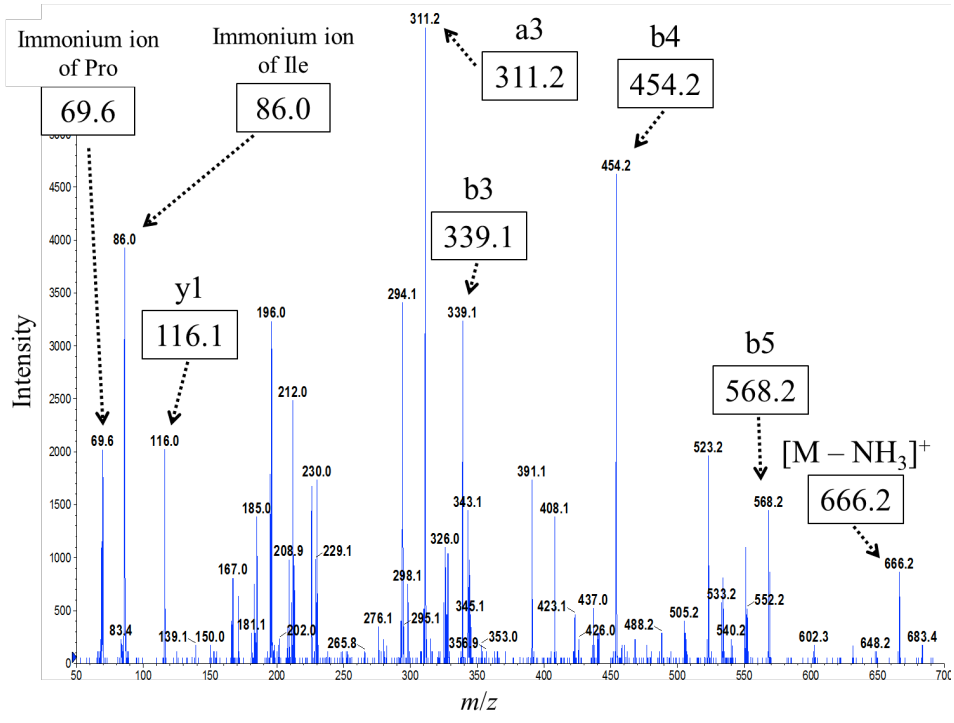


Supplementary figure 1. Electrospray ionization-mass spectrometry (ESI-MS) spectrum of the peak a, pyroGlu-Tyr, in Figure 5A.

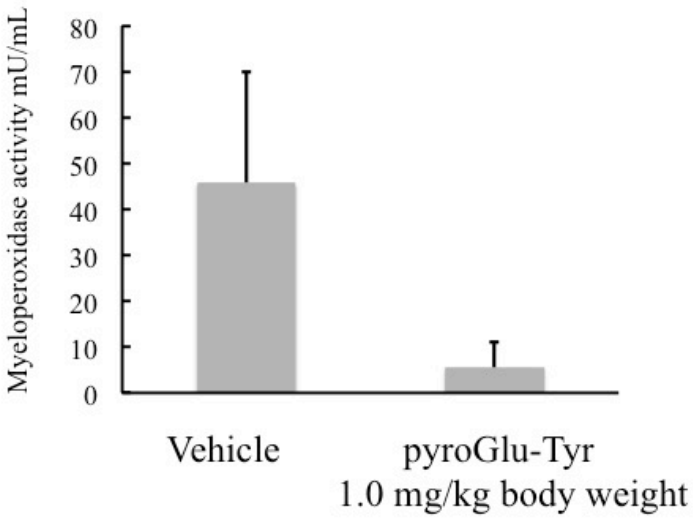


Supplementary figure 2. ESI-MS spectrum of the peak b, pyroGlu-Asn-Ile, in Figure 5A (upper) and ESI-tandem mass spectrometry (ESI-MS/MS) spectrum of the peak with an *m/z* value of 367.2 (lower).





Supplementary figure 3. ESI-MS spectrum of peak c, pyroGlu-Asn-Ile-Asp-Asn-Pro, in Figure 5A (upper) and ESI-MS/MS spectrum of the peak with an *m/z* value of 683.4 (lower).



Supplementary figure 4. Myeloperoxidase activity of the colonic extract of mice with colitis. Mice were received water (vehicle) or pyroGlu-Tyr.